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Transmissible spongiform encephalopathies (TSEs) are fatal untreatable neurodegenerative diseases associated with the accumulation of a disease-specific form of prion protein (PrP^{Sc}) in the brain. One approach to TSE therapeutics is the inhibition of PrP^{Sc} accumulation. Indeed, many inhibitors of PrP^{Sc} accumulation in scrapie-infected mouse neuroblastoma cells (ScN_2a) also have anti-scrapie activity in rodents. To expedite the search for potential TSE therapeutics, we have developed a high-throughput screening assay for PrP^{Sc} inhibitors using ScN_2a cells in a 96 well format. A library of 2000 drugs and natural products was screened in ScN_2a cells infected with RML (Chandler) or 22L scrapie strains. Seventeen had IC_{50} values of $\leq$ 1 μM against both strains. Several classes of compounds were represented in the 17 most potent inhibitors including naturally occurring polyphenols (e.g. tannic acid and tea extracts), phenothiazines, antihistamines, statins, and antimalarials. Several of the new PrP^{Sc} inhibitors cross the blood-brain barrier and thus have potential to be effective after TSE infection reaches the brain. Initial testing of inhibitors in transgenic mice was not effective, and this testing is described herein. Compounds were tested prior to ip inoculation and after ic inoculation.

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INTRODUCTION

This research is aimed at finding therapeutics against the Transmissible Spongiform Encephalopathies (TSEs) or prion diseases, which are currently untreatable. TSEs are neurodegenerative diseases associated with the deposition of a disease specific form of prion protein called PrP-res. This form of prion protein is a logical target for intervention as the basis for therapy. A cell-culture based assay has been developed with the aid of this grant that allows the high-throughput screening of molecules to find those with the best inhibitory activity against PrP-res. The first library screened was 2000 drugs and natural products and a number of those with promising activity. A number of the first promising candidates found were tested in transgenic mice inoculated intraperitoneally or intracranially with scrapie.

BODY

Research accomplishments associated with Task 1: To increase the throughput of a screen for anti-PrP^{Sc} activity in the scrapie-infected neuroblastoma cell model.

This task has been completed with the development of a high-throughput cell-culture assay to measure the inhibition of the formation of PrP^{Sc}. This assay was published in 2003 in the Journal of Virology (see appendix). This work was done between submission of the grant and award of funding.

Research accomplishments associated with Task 2: To develop a high-throughput cell-free system to measure the ability of compounds to interfere with PrP^{C} to protease-resistant PrP conversion.

This task has been accomplished in a low-throughput version (see testing of several compounds with this method in Journal of Virology in the appendix). Work will begin soon to attempt to improve the throughput of the assay to screen larger number of compounds.

Research accomplishments associated with Task 3: To screen libraries of compounds for anti-PrP^{Sc} activity.

The first library was screened using the high-throughput cell-culture method. These 2000 drugs and natural products yielded some promising inhibitors of PrP^{Sc} in cell-culture. The results from this screen of 2000 compounds are in the Journal of Virology article in the appendix. Work has begun on screening a new library of 7800 compounds.

Research accomplishments associated with Task 4: To test the compounds with the best activity in the anti-PrP^{Sc} screens in TSE-infected animals.

We have tested some promising PrP^{Sc} inhibitors in TSE-infected mice. Testing was done prophyactically against intraperitoneal inoculation and as a treatment against intracranial inoculation. The result from this first round of testing is in a 2004 Journal of General Virology article in the appendix. Testing will be ongoing in several cycles until we have tested all the best candidate molecules from the 2000 compound library. As new inhibitors are found they will be subsequently tested.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed a high-throughput dot blot assay to test compounds for inhibition of RML- and 22L-PrP-res in infected cells
- Screened a library of 2000 compounds for inhibitory activity and found 15 new inhibitors that can be tested in animals
- Tested the first batch of promising inhibitors in transgenic mice infected with scrapie

REPORTABLE OUTCOMES

Manuscipt:

Kocisko DA, Morrey JD, Race RE, Chen J, Caughey B. Evaluation of new cell culture inhibitors of protease-resistant prion protein against scrapie infection in mice. J Gen Virol. 2004;85:2479-2483.

Presentation:

Interactions between prion protein isoforms: The Kiss of Death? Presented to biotechnology department at Utah State University in March 2004.

CONCLUSIONS

We are happy with the progress we are making on this project. A robust high-throughput assay was developed and 2000 compounds were screened for anti-PrP-res activity against the two mouse strains of scrapie. The inhibitors found from this initial screening are currently being tested in scrapie-infected animals. The first round of testing in scrapie-infected animals was completed. We have begun screening a second larger library of 7800 compounds. As new inhibitors are found they can be tested in ongoing animal experiments.

The significance of this assay is that it can test a lot of compounds in a short time to find new potential therapies for TSEs. While the first round of testing found no beneficial activity in vivo, hopefully future tests will find that at least some of these inhibitors have activity in TSE-infected mice.

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Kocisko DA, Baron GS, Rubenstein R, Chen J, Kuizon S, Caughey B. New Inhibitors of Scrapie-Associated Prion Protein in a Library of 2,000 Drugs and Natural Products. J Virol. 2003;77:10288-10294.

Kocisko DA, Morrey JD, Race RE, Chen J, Caughey B. Evaluation of new cell culture inhibitors of protease-resistant prion protein against scrapie infection in mice. J Gen Virol. 2004;85:2479-2483.

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New Inhibitors of Scrapie-Associated Prion Protein Formation in a Library of 2,000 Drugs and Natural Products

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Transmissible spongiform encephalopathies (TSEs) are fatal, untreatable neurodegenerative diseases associated with the accumulation of a disease-specific form of prion protein (PrP) in the brain. One approach to TSE therapeutics is the inhibition of PrP accumulation. Indeed, many inhibitors of the accumulation of PrP associated with scrapie (PrP^{Sc}) in scrapie-infected mouse neuroblastoma cells (ScN_2a) also have antiscrapie activity in rodents. To expedite the search for potential TSE therapeutic agents, we have developed a high-throughput screening assay for PrP^{Sc} inhibitors using ScN_2a cells in a 96-well format. A library of 2,000 drugs and natural products was screened in ScN_2a cells infected with scrapie strain RML (Chandler) or 22L. Forty compounds were found to have concentrations causing 50% inhibition (IC_{50}s) of PrP^{Sc} accumulation of ≤10 μM against both strains. Seventeen had IC_{50}s of ≤1 μM against both strains. Several classes of compounds were represented in the 17 most potent inhibitors, including naturally occurring polyphenols (e.g., tannic acid and tea extracts), phenothiazines, antihistamines, statins, and antimalarial compounds. These 17 compounds were also evaluated in a solid-phase cell-free hamster PrP conversion assay. Only the polyphenols inhibited the cell-free reaction, and their IC_{50}s were near 100 nM. Several of the new PrP^{Sc} inhibitors cross the blood-brain barrier and thus have potential to be effective after TSE infection reaches the brain. The fact that many are either approved human drugs or edible natural products should facilitate their use in animal testing and clinical trials.

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases that include Creutzfeldt-Jakob disease, chronic wasting disease, scrapie, and bovine spongiform encephalopathy. These diseases are characterized by the accumulation of a form of prion protein (PrP) that is partially resistant to degradation by proteases (23). The infectious TSE agent is not fully understood but is surmised to contain the proteinase K (PK)-resistant aggregate of PrP (8). The occurrence of TSEs is associated with specific mutations in PrP, inoculation with infectious material, or apparently spontaneous onset (23). Currently, there are no therapies for TSEs, and the diseases are invariably fatal. Thus, it is important to identify compounds with therapeutic or prophylactic activity against these diseases.

The conversion of PrP from the normal, protease-sensitive, and nonaggregated form (PrP^{C}) to the aggregated and protease-resistant form associated with scrapie (PrP^{Sc}) or other TSEs (PrP^{TSE}) is a hallmark of the diseases. While the mechanism of neurodegeneration in TSEs is not clear, interactions between PrP^{C} and PrP^{TSE} seem to be important in the pathology of TSEs. Thus, the prevention of PrP^{TSE} formation and/or the elimination of existing PrP^{TSE} may be therapeutic (14, 22, 29).

Chronically scrapie-infected neuroblastoma cells (ScN_2a) have been used extensively as a model for studying TSEs (1). The cells produce PrP^{Sc}, permitting cellular processes associated with PrP^{Sc} production to be examined. ScN_2a cells have been used to study the effect of PrP mutations (16, 30), barriers to interspecies transmission (21, 25), PrP metabolism (5), and inhibitors of PrP^{Sc} formation (11). To expedite the screening of compounds for anti-PrP^{Sc} activity in cell cultures, slot blot and dot blot assays have been developed (24, 31). Many different types of compounds, such as sulfonated dyes (9), sulfated glycans (4), cyclic tetrapyrroles (7), polyene antibiotics (18), curcumin (6), lysosomotropic antimalarial compounds (11), phenothiazines (17), and polyamines (27), can inhibit PrP^{Sc} formation when added to the medium of these cells. In addition, several of these classes of inhibitors have prolonged the survival time of scrapie-infected animals when administered near the time of infection (3, 10, 12, 15, 22). Thus, ScN_2a cells provide a useful in vitro model for screening compounds for anti-TSE activity.

In the present study, we screened a commercially available library of drugs and natural products to find new candidates for therapeutic intervention against TSEs. The inhibition of PrP^{Sc} production was monitored in ScN_2a cells infected with scrapie strain RML (Chandler) (4) or 22L. PrP^{Sc} from cells plated in a 96-well format was assayed with a modification of the dot blot method of Rudyk et al. (24). Of the 2,000 compounds screened, 17 had concentrations causing 50% inhibition (IC_{50}s) of PrP^{Sc} accumulation of ≤1 μM against the RML and 22L strains. A number of these candidates are used for other

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indications in humans and would therefore be available for immediate clinical trials.

MATERIALS AND METHODS

Compound library. The library tested was The Spectrum Collection (MicroSource Discovery Inc., Groton, Conn.). The 2,000 compounds in the library are primarily Food and Drug Administration (FDA)-approved compounds or natural products. An alphabetical list of the compounds is available at the MicroSource Discovery website at www.msdiscovery.com/spect.html. The compounds are supplied as 10 mM solutions in dimethyl sulfoxide (DMSO).

Testing for PrP^{Sc} inhibitory activity in cell cultures. Approximately 20,000 RML (4)-infected or 22L-infected mouse neuroblastoma cells in 100 μl of medium were added to each well of a Costar 3595 flat-bottom 96-well plate with a low-evaporation lid (Corning Inc., Corning, N.Y.) prior to the addition of test compounds. 22L-infected cells were developed by reinfection of RML-infected mouse neuroblastoma cells cured by seven passages in 1 μg of pentosan polysulfate/ml of medium (2). The cured cells were reinfected by incubation with PrP^{Sc} purified from mouse brains infected with scrapie strain 22L. Others have reported the susceptibility of mouse neuroblastoma cells to 22L infection (20). Neuroblastoma cells reinfected with 22L have stably expressed PrP^{Sc} for over 100 passages. The cells were allowed to settle for 4 h before test compounds were added.

The 10 mM solutions of test compounds were diluted in DMSO and then in phosphate-buffered saline (PBS) prior to being introduced to the cell medium. Five-microliter solutions were added to the cell medium. DMSO concentrations in the cell medium were never higher than 0.5% (vol/vol). After a compound was added, the cells were incubated for 5 days at 37°C in a CO_2 incubator before being lysed.

Prior to cell lysis, the cells were inspected by light microscopy for toxicity, bacterial contamination, and density compared to controls. After removal of the cell medium, 50 μl of lysis buffer was added to each well. Lysis buffer was composed of 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 5 mM Tris-HCl (pH 7.4 at 4°C), 5 mM EDTA, and 150 mM NaCl. At 5 min after the addition of lysis buffer, 25 μl of PK (0.1 mg/ml; Calbiochem) in Tris-buffered saline (TBS) was added to each well and incubated at 37°C for 50 min. A total of 225 μl of 1 mM Pefabloc (Boehringer Mannheim) was added to each well to inhibit PK activity. A total of 250 μl of 1 mM Pefabloc was added to samples that were not PK treated.

High-throughput measurement of PrP^{Sc} by a dot blot procedure. The dot blot procedure used is a streamlined version of that developed by Rudyk et al. (24). A 96-well dot blot apparatus (Schleicher & Schuell) was set up with a 0.45-μm-pore-size polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore), and each dot was rinsed with 500 μl of TBS. Under vacuum, the lysed and PK-treated samples were added to the apparatus over the PVDF membrane and rinsed with 500 μl of TBS. The PVDF membrane was removed and covered with 3 M GdnSCN (Fluka) for 10 min at ambient temperature. GdnSCN was removed by five PBS rinses, and the membrane was blocked with 5% (wt/vol) milk–0.05% (vol/vol) Tween 20 (Sigma) in TBS (TBST-milk) for 30 min. An appropriate dilution of monoclonal antibody 6B10, an immunoglobulin G2a antibody reactive against mouse, hamster, elk, and sheep PrP in immunoblotting assays and enzyme-linked immunosorbent assays (data not shown), or 8 μg of purified anti-PrP mouse monoclonal antibody 6H4 (Prionics) in 15 ml of TBST-milk was incubated with the membrane for 60 min. After TBST rinsing, a solution of ~500 ng of an alkaline phosphatase-conjugated goat anti-mouse antibody (Zymed) in 15 ml of TBST-milk was added and incubated for 45 min. After additional TBST rinsing, the membrane was treated with an enhanced chemifluorescence agent (Amersham) for 10 min, allowed to dry, and then scanned with a Storm Scanner (Molecular Dynamics). The intensity of the PrP^{Sc} signal from each well was quantitated by using ImageQuant software (Molecular Dynamics). Each 96-well plate had six untreated control wells and six wells treated with curcumin, a known PrP^{Sc} inhibitor in RML-infected ScN_2a cells (6).

Solid-phase PrP conversion assay. In brief, for the solid-phase PrP conversion assay (18), a 100-ng suspension of hamster scrapie strain 263K PrP^{Sc} in 40 μl of PBS was added to wells of a 96-well plate and air dried to promote adherence of the protein to the surface. The wells were then blocked with 2% bovine serum albumin in PBS. This solution was removed, and another solution, containing ~20,000 cpm of hamster ^{35}S-labeled PrP^{C} with or without potential inhibitors, was added and incubated at 37°C for 48 h. The ^{35}S-labeled PrP^{C} solution was removed, and the wells were washed. PK (20 μg/ml) was added to the wells and then removed after 1 h to digest unconverted but bound ^{35}S-labeled PrP^{C}. The protein in the wells was eluted by boiling in sodium dodecyl sulfate sample buffer and scintillation counted. To obtain the relative percent conversion, the measured counts in PrP^{Sc} wells less the counts in bovine serum albumin-blocked wells lacking PrP^{Sc} were compared to the total ^{35}S-labeled PrP^{C} counts added to the wells.

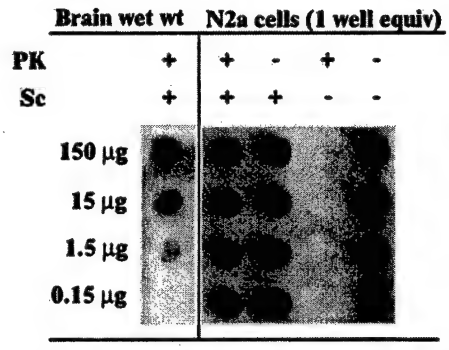

FIG. 1. Dot blot of brain-derived PrP^{Sc} and ScN_2a cell-derived PrP^{C} and PrP^{Sc}. The wells shown are from a single membrane visualized with primary antibody 6H4. The samples in the first lane contain the indicated brain wet-weight (wet wt) equivalents in a lysate from a hamster clinically ill from infection with scrapie strain 263K. The second and third lanes from the left contain lysates from RML-infected ScN_2a cells (one well equivalent). The fourth and fifth lanes contain lysates from uninfected N_2a cells. PrP^{C} from uninfected cells was detected without any PK treatment.

RESULTS

High-throughput screen for PrP^{Sc} inhibitors. To facilitate the screening of large numbers of compounds for the inhibition of PrP^{Sc} accumulation, we developed a high-throughput test using ScN_2a cell cultures in combination with a rapid dot blot assay for PrP^{Sc} (scrapie cell dot blot [SCDB] assay). PrP^{Sc} from one well of ScN_2a cells in a 96-well plate was readily detectable by the SCDB assay (Fig. 1). Without PK treatment, PrP^{C} from uninfected N_2a cells was also readily detectable, but PK treatment eliminated this signal. Dilutions of scrapie-infected brain homogenates indicated that the PrP^{Sc} signal intensity from one well of cells fell between that from samples with 1.5 and 15 μg of brain wet-weight equivalents (Fig. 1) in a linear response range of the PrP^{Sc} SCDB assay (data not shown). Similar results were obtained with anti-PrP monoclonal antibodies 6H4 (Fig. 1) and 6B10 (data not shown). A typical dot blot from the SCDB assay with antibody 6B10 is shown in Fig. 2.

Screening of a 2,000-compound library. The Spectrum Collection, a library of 2,000 drugs and natural products, was screened for PrP^{Sc} inhibitory activity with the SCDB assay. The identities of the compounds were not revealed to the investigator until screening was completed. A flowchart of the screening sequence is shown in Fig. 3. The compounds were screened initially at 10 μM against RML-infected cells. Approximately 70% of the compounds showed less than 50% inhibition of PrP^{Sc} formation at 10 μM in these cells and were not evaluated further. Approximately 20% (398) of the compounds were cytotoxic at 10 μM and were tested again at 1 μM. A smaller group of 246 compounds inhibited RML PrP^{Sc} accumulation by more than 50% at 10 μM without observed toxicity. These 246 compounds were tested further at 10 μM in ScN_2a cells infected with scrapie strain 22L, and 40 of them were found to reduce PrP^{Sc} accumulation by ≥50% (35 compounds with IC_{50}s of between 1 and 10 μM are shown in Fig. 4). These 40

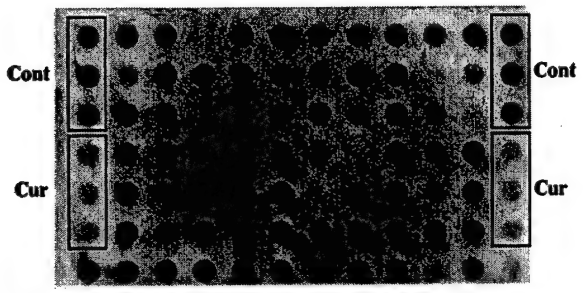

FIG. 2. Partial 96-well dot blot showing the PK-resistant PrP signal visualized with primary antibody 6B10. Signals from untreated control (Cont) cells and curcumin-inhibited (Cur) cells are indicated. The latter were incubated in the presence of 10 μM curcumin, a known inhibitor of PrP^{Sc} in RML-infected cells (6). Other dots represent signals from ScN_2a cells after incubation with 10 μM concentrations of various compounds. Some of these spots have an intensity comparable to that of controls, indicating no inhibition of PrP^{Sc} formation. Others that are less intense were due to compounds with various inhibitory strengths or toxicities.

compounds were then tested at 1 μM against both RML- and 22L-infected cells, revealing 5 compounds with IC_{50}s of ≤1 μM against both strains. Twelve additional inhibitors fitting these criteria were discovered when the 398 compounds cytotoxic at 10 μM were tested at 1 μM against both RML- and 22L-infected cells. Thus, of the 2,000 compounds screened, 17 had an IC_{50} of ≤1 μM against both scrapie strains without observed toxicity (Fig. 5).

For compounds to pass through the screen described in Fig. 3, the cells had to grow from low density to confluence and thrive in the presence of test compounds. However, as an additional test for potential cytotoxicity, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell viability assay was performed with the 17 most potent inhibitors (19). The assay was done in duplicate after 3 and 4 days of incubation with the 17 compounds at 1 μM. The percentage of cell viability with all compounds tested in the MTT assay was in the range of 90 to 129% of controls on both days. In contrast, with aklavine and celastrol, which had visually obvious cytotoxicity, 2 to 9% cell viability was obtained on both days. The inhibition of PrP^{Sc} accumulation by these 17 compounds in the SCDB assay was confirmed in six separate experiments. Subsequent testing of these 17 compounds at 500, 100, and 10 nM revealed that tannic acid had an IC_{50} of ~100 nM, whereas the other 16 compounds had IC_{50}s of between 100 nM and 1 μM (Fig. 5).

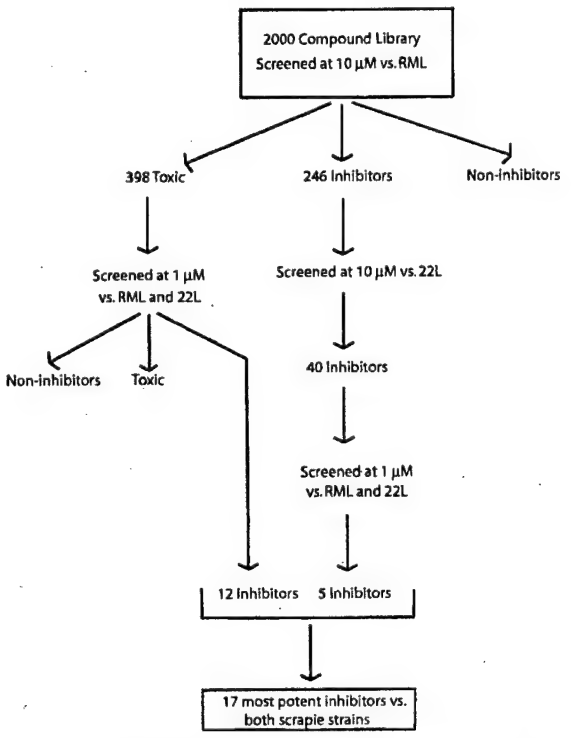

FIG. 3. Flowchart of the screening of The Spectrum Collection compound library.

Of the 17 most potent inhibitors in The Spectrum Collection that were active against both scrapie strains, two, quinacrine and lovastatin, were identified previously as PrP^{Sc} inhibitors (11, 28). The remaining 15 compounds are novel inhibitors representing multiple classes of drugs or natural products, including polyphenols (e.g., tea and tree gall extracts), antimalarial compounds, antihistamines, phenothiazine analogs (e.g., antipsychotics), statins (hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitors), and others, as indicated in Fig. 5.

Test of inhibition of cell-free PrP conversion. To test for direct effects on PrP conversion, the 17 most potent inhibitors were added to a solid-phase cell-free conversion (SP-CFC) reaction in which hamster PrP^{Sc} is used to induce the conversion of radiolabeled hamster PrP^{C} to a PrP^{Sc}-like PK-resistant state (18a). Three polyphenols, tannic acid, katacine, and 2′,2′″-bisepigallocatechin digallate, inhibited the SP-CFC reaction, with an IC_{50} of approximately 100 nM (Fig. 6). The other 14 compounds were not inhibitory in the SP-CFC reaction at concentrations up to 100 μM (data not shown).

Test for destabilization of preexisting PrP^{Sc}. To search for compounds that can destabilize preexisting PrP^{Sc}, compounds with IC_{50}s of ≤10 μM were incubated at 250 μM with ScN_2a cell lysates for 24 h at 37°C to determine whether they could increase the PK sensitivity of PrP^{Sc}. However, even at a concentration at least 25 times its IC_{50}, no compound was able to increase the PK sensitivity of PrP^{Sc} (data not shown).

DISCUSSION

The high-throughput SCDB assay has greatly expedited our search for new, potentially therapeutic inhibitors of PrP^{Sc} accumulation. Clearly, one cannot expect that all compounds selected as potent inhibitors with this in vitro screen will prove to be effective anti-TSE drugs in vivo. However, given that a majority of the different classes of compounds that were previously identified as potent inhibitors by use of RML-infected ScN_2a cells have prophylactic efficacy against scrapie in vivo, we expect the SCDB assay will be valuable in the initial screening of potential drugs from large compound libraries. The additional use of ScN_2a cells infected with the 22L strain of scrapie in the SCDB assay helps to identify compounds with broader, less strain-dependent inhibitory activity. Adaptations of the SCDB assay for use with TSE-infected cell cultures from

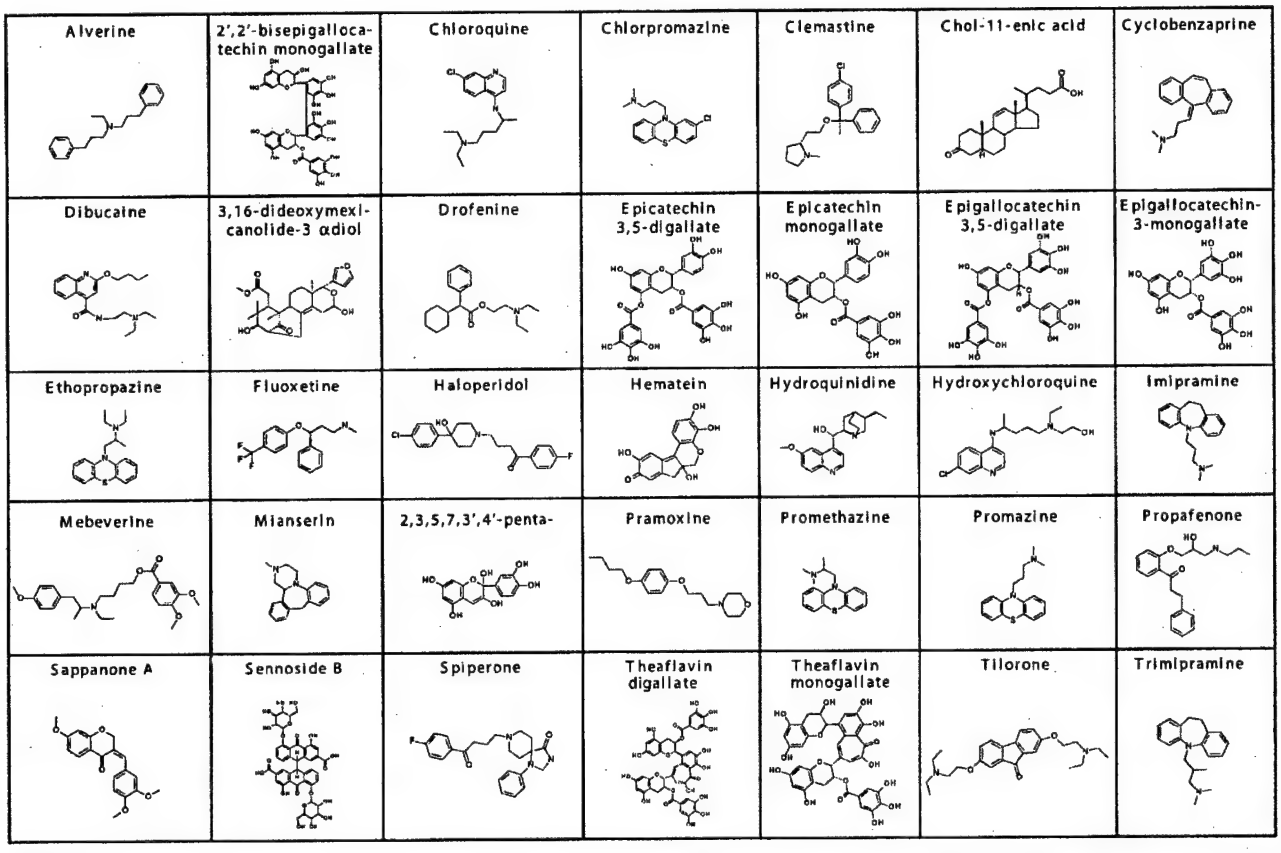

FIG. 4. Structures of compounds in The Spectrum Collection with IC_{50}s of >1 and ≤10 μM against both the RML and 22L scrapie strains, listed in approximate alphabetical order. 2,3,5,7,3',4'-penta-, 2,3,5,7,3',4'-pentahydroxyflavan.

other species should increase the value of the assay for predicting efficacy against TSE diseases of humans and livestock.

In our screening of the 2,000 compounds of The Spectrum Collection, both new and old inhibitors were identified. Of the 17 most potent inhibitors in the library with activity against the RML and 22L mouse scrapie strains (Fig. 5), 15 were new, whereas quinacrine and lovastatin were already known as PrP^{Sc} inhibitors in scrapie-infected cell cultures (11, 28). Other previously identified inhibitors, such as chloroquine (11) and promazine, promethazine, and chlorpromazine (17), also inhibited PrP^{Sc} accumulation in the SCDB assay screening (Fig. 4) but were not among the 17 most potent and strain-independent compounds in the library. The fact that several previously known inhibitors were selected by our blind screening of a large compound library inspires confidence in the utility of the SCDB assay.

Polyphenol inhibitors. Numerous polyphenols were selected as PrP^{Sc} inhibitors against both strains of mouse scrapie in the SCDB assay. Tannin (tannic acid), the most potent inhibitor found, is a relatively nontoxic constituent of foods such as tea, red wine, beer, and nuts. 2',2'''-Bisepigallocatechin digallate is also a component of tea, and katacine is another naturally occurring polyphenol antioxidant. Relatively few studies have been done on the bioavailability of the polyphenols from tea extracts, but significant oral absorption has been shown in humans (32). While at first glance these water-soluble compounds might not be considered likely to cross the blood-brain barrier, radiolabeled epigallocatechin gallate, another tea extract polyphenol, has been detected in mouse brains after oral administration (26). A number of other polyphenols, including epigallocatechin 3,5-digallate and epicatechin monogallate, were included in the group with IC_{50}s of between 1 and 10 μM (Fig. 4). The naturally occurring polyphenols represent a part of the normal human diet and are relatively nontoxic. Even if the ability of these compounds to cross the blood-brain barrier is questionable, they may be useful as prophylactic agents against peripheral infections or as TSE decontaminants.

Not all polyphenols tested were PrP^{Sc} inhibitors. Epicatechin and epigallocatechin, with molecular weights of about 300, were ineffective, although they represent portions of larger polyphenol molecules that were effective, such as epigallocatechin 3,5-digallate. While most of the polyphenol inhibitors were larger than 350 Da, a similar polyphenol with a molecular weight of 304, 2,3,5,7,3',4'-pentahydroxyflavan, was an inhibitor. This molecule is more conjugated and planar than epicatechin (Fig. 7). Although these results indicate that minor structural differences can have dramatic effects on polyphenol efficacy, further study is needed to clarify the structure-activity relationships.

Malaria drugs. Quinacrine and other antimalarial compounds have been reported to inhibit PrP^{Sc} formation in cell-based assays (11, 17). Quinacrine was reported to have an IC_{50}

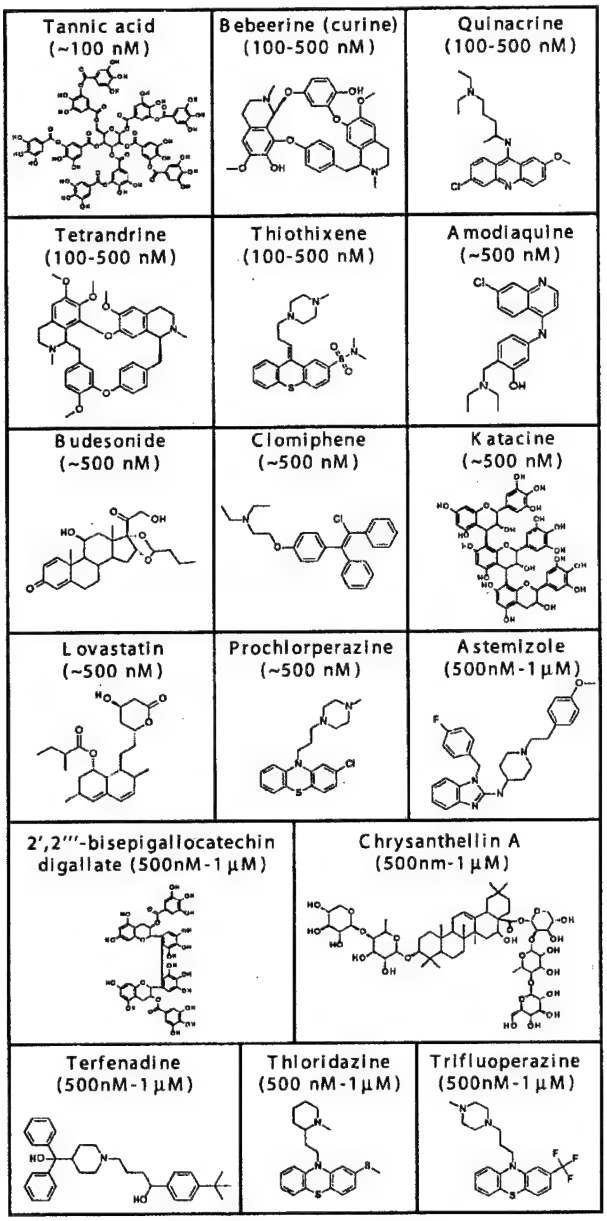

FIG. 5. Structures of compounds in The Spectrum Collection with IC_{50}s of ≤1 μM against both the RML and 22L strains of scrapie. Compounds are arranged from low to high approximate IC_{50}s.

of 400 nM, in good agreement with our present results. However, quinacrine has not shown any long-term benefit against Creutzfeldt-Jakob disease in preliminary clinical trials in humans (13). Since drug bioavailability, transport, and metabolism can depend markedly on structural details, it is possible that the other antimalarial compounds that were identified in this study are more effective than quinacrine in vivo. For instance, amodiaquine and bebeerine were among the 17 most potent inhibitors. Amodiaquine is a 4-aminoquinoline analog of chloroquine that is currently used as an antimalarial drug, but it was a stronger inhibitor of PrP^{Sc} formation in the SCDB assay. Chloroquine had an IC_{50} between 1 and 10 μM, in good

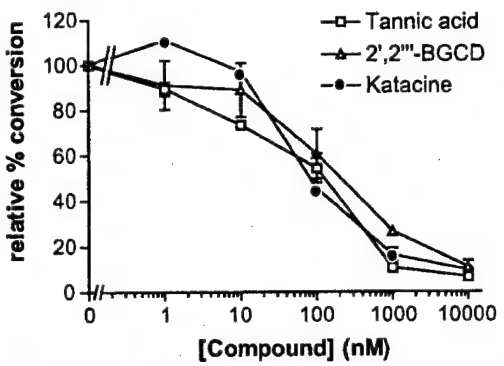

FIG. 6. Inhibition of solid-phase cell-free PrP conversion by polyphenols. The conversion relative to that in control reactions is plotted against the concentration of polyphenol added to the reaction. 2',2'''-BGCD, 2',2'''-bisepigallocatechin digallate.

agreement with a previous study (11). The closely related compounds hydroxychloroquine and hydroquinidine had similar IC_{50}s. Bebeerine (curine) is a bisbenzylisoquinoline alkaloid naturally produced from the root bark of *Chondrondendron platyphyllum*. Another naturally produced bisbenzylisoquino-

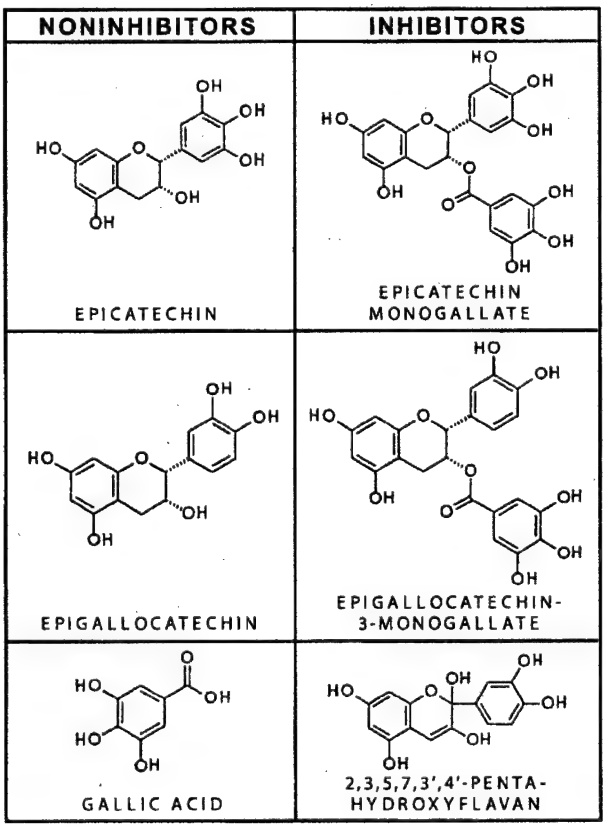

FIG. 7. Structural comparisons of inhibitory and noninhibitory polyphenols. Epicatechin and epigallocatechin were not inhibitors until the addition of a gallate, which was not an inhibitor on its own. Compared to epicatechin, the inhibitor 2,3,5,7,3',4'-pentahydroxyflavan has one additional conjugated double bond and an additional hydroxyl group. The double-ring system in the flavan should be more planar than the corresponding rings in epicatechin.

line alkaloid, tetrandrine, was also a potent inhibitor. Although not known to be antimalarial, this compound is a nonselective Ca^{2+} channel blocker derived from a Chinese medicinal herb, *Stephenia tetrandra* S. Moore, and has been used to treat hypertension and autoimmune disorders in traditional Chinese medicine.

Antihistamines. The antihistamines astemizole and terfenadine were both among the most potent PrP^{Sc} inhibitors. These compounds are known to be poor at crossing the blood-brain barrier, a fact which may limit their therapeutic usefulness against TSEs. These antihistamines have been used extensively in humans but are currently not marketed in the United States because of a concern for serious, but rare, cardiovascular toxicity and the availability of safer alternatives.

Phenothiazine derivatives and analogs. The phenothiazine derivatives chlorpromazine, promazine, and promethazine inhibited PrP^{Sc} accumulation, in agreement with another study (17). However, our screen identified several more potent phenothiazine inhibitors, including the FDA-approved antipsychotics thioridazine, trifluoperazine, and prochlorperazine. The most potent group of 17 inhibitors identified in this study also included the FDA-approved antipsychotic thiothixene, which is a phenothiazine structural analog. These phenothiazine derivatives and analogs penetrate the blood-brain barrier, a feature that should be beneficial in treating TSEs.

Other inhibitors. Lovastatin is an FDA-approved hepatic hydroxymethyl glutaryl coenzyme A reductase inhibitor that reduces blood cholesterol levels and is known to cross the blood-brain barrier. Its inhibition of PrP^{Sc} accumulation at 500 nM agrees with a previous study (28) and places it among the best inhibitors. Budesonide is a steroid derivative approved by the FDA to treat asthma, chrysanthellin A is a naturally produced steroidal glycoside, and clomiphene is the FDA-approved treatment of choice for anovulatory infertile women with polycystic ovary syndrome.

Inhibition of cell-free PrP conversion. The SP-CFC reaction monitors direct hamster PrP interactions. Because there presumably are therapeutic targets besides PrP conversion for the TSEs in vivo, a compound could be effective in scrapie-infected cells and animals without being effective in the SP-CFC assay. For example, quinacrine was an effective PrP^{Sc} inhibitor in the SCDB assay but was not effective at inhibiting the SP-CFC reaction. Quinacrine is a lysosomotropic amine and may function by altering endosomal or lysosomal microenvironments (11). Another example is lovastatin, which is thought to inhibit PrP^{Sc} formation indirectly by depleting cellular cholesterol (28), consistent with its inability to block the SP-CFC reaction. Indeed, a majority of the 17 most potent inhibitors in the SCDB assay were unable to block the SP-CFC reaction. Another possible explanation for the discordance between the SCDB and SP-CFC assays is the species specificity of interactions with PrP isoforms. The SCDB and SP-CFC assays involve mouse and hamster PrP molecules, respectively. Regardless, the three polyphenols were potent inhibitors in both types of assays and thus appear to be direct inhibitors of PrP conversion. Although we do not anticipate that the screening of compound libraries with the SP-CFC assay alone would be as predictive of in vivo efficacy as the SCDB assay, we have shown that the cell-free assay can be used to obtain mechanistic insights into whether inhibitors identified in the SCDB assay act via direct or indirect mechanisms.

Conclusion. This screening has identified new compounds and classes of compounds that are effective PrP^{Sc} inhibitors against two scrapie strains in cell cultures. The naturally occurring polyphenols were also effective inhibitors of cell-free PrP conversion. Barring hamster and mouse PrP species differences, these results suggest that the polyphenols inhibit PrP^{Sc} formation through direct PrP interactions, whereas the other inhibitors may work indirectly. Among the list of the 17 best inhibitors are FDA-approved compounds and dietary constituents that should be acceptable for testing in infected animals and humans. The fact that a number of the new inhibitors are known to cross the blood-brain barrier makes them attractive as potential anti-TSE therapeutic agents and distinguishes them from many previously identified PrP^{TSE} inhibitors.

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Short Communication

Evaluation of new cell culture inhibitors of protease-resistant prion protein against scrapie infection in mice

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In vitro inhibitors of the accumulation of abnormal (protease-resistant) prion protein (PrP-res) can sometimes prolong the lives of scrapie-infected rodents. Here, transgenic mice were used to test the *in vivo* anti-scrapie activities of new PrP-res inhibitors, which, because they are approved drugs or edible natural products, might be considered for clinical trials in humans or livestock with transmissible spongiform encephalopathies (TSEs). These inhibitors were amodiaquine, thioridazine, thiothixene, trifluoperazine, tetrandrine, tannic acid and polyphenolic extracts of tea, grape seed and pine bark. Test compounds were administered for several weeks beginning 1–2 weeks prior to, or 2 weeks after, intracerebral or intraperitoneal 263K scrapie challenge. Tannic acid was also tested by direct preincubation with inoculum. None of the compounds significantly prolonged the scrapie incubation periods. These results highlight the need to assess TSE inhibitors active in cell culture against TSE infections *in vivo* prior to testing these compounds in humans and livestock.

Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative diseases that affect mammals. Examples of TSEs include sheep scrapie, bovine spongiform encephalopathy, chronic wasting disease of cervids and Creutzfeldt–Jakob disease (CJD) in humans. TSEs are associated with the accumulation of an abnormal and protease-resistant aggregate of prion protein (PrP) known as PrP-res or PrP^{Sc} (Caughey & Lansbury, 2003). The exact makeup of the infectious particle of the TSEs is not clear, but infectious preparations are composed mainly of PrP-res, which accumulates primarily in the brains of affected individuals. All TSEs are fatal and there is no known cure for these diseases.

Because PrP-res is associated with disease and infectivity, it has been a target of therapeutic intervention for TSEs (Aguzzi *et al.*, 2001; Dormont, 2003; Brown, 2002). Murine N2a cells chronically infected with the RML (Chandler) strain of scrapie (Race *et al.*, 1988) have been used widely to test compounds for their ability to inhibit PrP-res formation (Caughey *et al.*, 1999; Beranger *et al.*, 2001; Kocisko *et al.*, 2003). Many compounds that inhibit PrP-res in cell culture have also delayed the onset of TSEs in animal models, but none has been curative. These compounds include porphyrins and phthalocyanins (Caughey *et al.*, 1998; Priola *et al.*, 2000), polyene antibiotics (Dormont, 2003), Congo red (Caughey & Race, 1992; Ingrosso *et al.*, 1995), suramin (Gilch *et al.*, 2001), sulfated glycans and other polyanions (Ehlers & Diringer, 1984; Kimberlin & Walker, 1986; Farquhar & Dickinson, 1986; Caughey & Raymond, 1993; Birkett *et al.*, 2001; Schonberger *et al.*, 2003; Gabizon *et al.*, 1993). We recently used N2a cell cultures infected with either the RML or the 22L strains of scrapie to screen PrP-res inhibitors from a library of 2000 drugs and natural products (Kocisko *et al.*, 2003).

In this study we tested a number of the most potent of these new cell culture PrP-res inhibitors against scrapie infection in transgenic mice (Tg7). Tg7 mice produce no mouse PrP, but express approximately 4- to 8-fold higher levels of hamster PrP than do hamsters. They have a short disease incubation period of ~45–50 days after intracerebral (i.c.) inoculation with a high dose of the 263K strain of hamster scrapie (Race *et al.*, 2000; Priola *et al.*, 2000).

Compounds were administered either to treat an established infection or to test for prophylaxis. To test for activity against an established infection, compound administration started 2 weeks after i.c. scrapie inoculation and continued for 5–6 weeks. The 2-week period after i.c. inoculation

allowed time for the disease to progress before the compound was administered. To test for prophylaxis, administration of a compound began 2 weeks before and continued for 4 weeks after intraperitoneal (i.p.) scrapie inoculation. The rationale was to have a compound approaching a steady-state level in the mouse, enabling it to block a peripheral inoculation of scrapie infectivity from being established in the brain. The treatment following inoculation would allow time for the animal potentially to clear infectious material while the compound prevented further formation of PrP-res.

Compounds were administered either as an i.p. injection or in the drinking water. For i.p. injections, compounds were dissolved or suspended in an appropriate buffer and the dose volume was 10 ml kg^{-1}. Injections were given three times per week, on Monday, Wednesday and Friday. Solutions of compounds in drinking water were made to yield the desired dose based on the mean daily consumption of water by mice, 15 ml (100 g body wt)$^{-1}$. A solution of compound in the drinking water was the sole source of water for the mice during the dosing period. All 263K scrapie brain homogenates made up for inoculation in these studies were in physiological buffer supplemented with 2 % fetal bovine serum. Different control groups are presented because testing was not done all at once and mice were inoculated with different homogenate preparations. In these studies, Tg7 mice were euthanized when clinical signs of scrapie were present, which included ruffled fur, lethargy, ataxia and weight loss. All procedures were approved by the Institution's Animal Care and Use Committee and were designed to minimize the animals' pain and distress. Animals that died from causes other than scrapie, such as from inoculation, dosing and anaesthetizing procedures, have been excluded from the data.

Compounds evaluated in animals had IC_{50} (concentration of a compound inhibiting half of the production of PrP-res) values of $\leqslant 1$ μM against both the RML and the 22L scrapie strains in cell culture. Since the *in vivo* testing involved hamster 263K scrapie, it was felt that compounds that inhibited multiple strains of mouse scrapie had a better chance of showing efficacy against PrP from another species. The inhibitors tested had been identified previously (Kocisko *et al.*, 2003) except for polyphenolic extracts of grape seed and pine bark (data not shown). In addition to their history of use in humans, the anti-psychotic drugs thioridazine, thiothixene and trifluoperazine were also selected for testing because they are known to cross the blood–brain barrier of humans. Amodiaquine is an inexpensive anti-malarial drug that has been used extensively in humans. The polyphenol tannic acid, which is contained in many foods, was the most potent inhibitor in our test set with an IC_{50} of ~100 nM in both the scrapie-infected neuroblastoma cells and a solid-phase cell-free hamster 263K conversion assay (Kocisko *et al.*, 2003). A tea extract containing ~55 % epigallocatechin monogallate and other polyphenols was also tried because of its relatively low toxicity and use as a human food. Finally, tetrandrine, a Chinese herbal medicine with anti-malarial activity, was tested. Generally, the highest known tolerated dose of a compound in mice was given to maximize the chance of seeing an effect. For instance, 5 mg thioridazine kg^{-1} dosed i.p. was used in this trial because 10 mg kg^{-1} i.p. is not tolerated (Burke *et al.*, 1990). In our experiments, 10 mg trifluoperazine kg^{-1} was mildly toxic but was tolerated, and 4500 mg tannic acid kg^{-1} per day was not tolerated but 3000 mg kg^{-1} per day had no apparent toxicity.

Table 1 contains the incubation period of each individual Tg7 mouse after i.c. inoculation of 263K scrapie brain homogenate and administration of compounds. No compound used as a treatment against established infection after i.c. inoculation significantly extended incubation periods. Nor was any compound protective when administered for a week prior to i.c. inoculation.

Prophylaxis tests with a number of compounds were also done on animals infected by i.p. inoculation to test for inhibition of the spread of infection from the periphery, where most natural infections initiate. A lack of effect against i.c. inoculation may be due to low brain penetration for some compounds such as tannic acid, epigallocatechin monogallate in tea, and other naturally occurring polyphenols from pine bark or grape seed. In addition, one of the anti-psychotic drugs that does cross the blood–brain barrier, trifluoperazine, was also tried in this type of test to see if it would perform better against an i.p. inoculation compared with an i.c. inoculation. None of these drug treatments showed any efficacy against an i.p. inoculation (Table 2).

In addition to being a potent PrP-res inhibitor *in vitro*, tannic acid is appealing as a potential drug because of its relatively low oral toxicity and low cost. However, with a molecular mass of ~1700 Da, tannic acid would be unlikely to cross the blood–brain barrier in significant quantity and its bioavailability via the oral route in the mouse may not be high enough to be effective. In agreement with this, previously mentioned prophylaxis and therapeutic tests in scrapie-infected Tg7 mice with orally administered tannic acid demonstrated no benefit. Therefore, we tried direct incubation of tannic acid with infectious brain homogenate. Solutions of tannic acid at 10 mM, 1 mM and 10 μM in the presence of 5 % scrapie-infected brain homogenate were tested for their ability to reduce infectivity. After incubation at 37 °C for 2 h, the solutions were diluted 5000-fold to 0·001 % brain homogenate and then inoculated i.c. into Tg7 mice to assess infectivity. The results in Table 3 indicate that incubation of infectious material with tannic acid had no significant effect on scrapie incubation period.

There are many reasons why a given compound that is effective *in vitro* might not show efficacy in an *in vivo* test against scrapie infection. The pharmacokinetics and metabolism of these compounds in mice might be unfavourable. The drug concentration attained at an active site using the highest tolerated dose may not be high enough for long

Table 1. Treatment of Tg7 mice inoculated with 263K scrapie

M, Monday; W, Wednesday; F, Friday; inoc., inoculation; homog., homogenate; wk, week.

Compound	Dose (mg kg^{-1})	Dosing regimen	Scrapie inoculation	Incubation periods (days)	Mean ± SD
None			50 µl 0·01 % brain homog. i.c.	59, 63, 63, 63, 63, 56, 63, 64	61·8 ± 2·8
Amodiaquine	50 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0·01 % brain homog. i.c.	56, 63, 59, 60, 63	60·2 ± 2·9
Thioridazine	5 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0·01 % brain homog. i.c.	59, 63, 63, 58, 56, 59, 63, 63, 59	60·3 ± 2·7
Thiothixene	5 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0·01 % brain homog. i.c.	58, 56, 58, 58, 66, 58, 63, 65, 63, 58, 56, 63	60·2 ± 3·6
Trifluoperazine	10 i.p.	M, W, F for 5 wks starting 2 wks after inoc.	50 µl 0·01 % brain homog. i.c.	65, 63, 65, 72, 59, 63, 63, 63, 63, 64	64·0 ± 3·3
None			50 µl 0·001 % brain homog. i.c.	62, 69, 65, 66, 69, 69, 71, 66, 71, 65, 62, 62	66·4 ± 3·4
Tetrandrine	50 i.p.	M, W, F for 6 wks starting 2 wks after inoc.	50 µl 0·001 % brain homog. i.c.	64, 77, 69, 72, 64, 61	67·8 ± 6·0
Tannic acid	1500 per day in drinking water	Continuously for 6 wks starting 2 wks after inoc.	50 µl 0·001 % brain homog. i.c.	66, 63, 64, 68, 63, 64, 62, 64	64·3 ± 1·9
Polyphenolic tea extract	1500 per day in drinking water	Continuously for 6 wks starting 2 wks after inoc.	50 µl 0·001 % brain homog. i.c.	76, 79, 66, 73, 69, 57	70·0 ± 7·9
None			50 µl 0·01 % brain homog. i.c.	71, 71, 71, 77, 71, 71, 79, 71	72·8 ± 3·3
Amodiaquine	50 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0·01 % brain homog. i.c.	74, 74, 77, 74, 77, 81, 79, 74	76·3 ± 2·7
Thioridazine	5 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0·01 % brain homog. i.c.	74, 77, 71, 71, 77, 74, 71, 71	73·3 ± 2·7
Thiothixene	5 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0·01 % brain homog. i.c.	84, 77, 71, 74, 84, 71, 80	77·3 ± 5·6
Trifluoperazine	10 i.p.	M, W, F for 6 wks starting 1 wk prior to inoc.	50 µl 0·01 % brain homog. i.c.	74, 71, 71, 77, 84, 77, 71	75·0 ± 4·8

Table 2. Prophylaxis of Tg7 mice against 263K scrapie

All mice were infected with scrapie by i.p. inoculation with 50 µl 1 % brain homogenate. M, Monday; W, Wednesday; F, Friday; wk, week.

Test compound/preparation	Dose (mg kg^{-1})	Dosing regimen	Incubation periods (days)	Mean ± SD
None			101, 81, 127, 88, 97, 83, 88, 99	95·5 ± 14·7
Tannic acid	1500 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	89, 94, 87, 90, 89, 90, 96, 73	88·5 ± 6·9
Tannic acid	3000 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	81, 102, 87, 92, 88, 93	90·5 ± 7·1
Polyphenolic tea extract	1500 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	77, 105, 90, 77, 96, 77, 81, 75	84·8 ± 11·0
Polyphenolic grape seed extract	2250 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	80, 91, 100, 95, 73, 108, 97, 98, 90, 71	90·3 ± 12·1
Polyphenolic pine bark extract	2250 per day in drinking water	Continuously for 6 wks starting 2 wks prior to inoc.	90, 87, 95, 91, 87, 71, 73, 91, 90, 83	85·8 ± 7·9
Trifluoperazine	10 i.p.	M, W, F for 6 wks starting 2 wks prior to inoc.	90, 91, 90, 104, 99, 101, 103	96·9 ± 6·3

Table 3. Tannic acid as a potential 263K scrapie disinfectant

[Tannic acid], tannic acid concentration incubated with brain homogenate (homog.).

[Tannic acid]	Scrapie inoculation (i.c.)	Incubation periods (days)	Mean ± SD
None	50 µl 0·001 % brain homog.	62, 64, 63, 68, 69, 64	65·0±2·8
10 µM	50 µl 0·001 % brain homog. in 2 nM tannic acid	72, 69, 59, 60, 61, 62, 61, 62	63·3±4·7
1 mM	50 µl 0·001 % brain homog. in 200 nM tannic acid	63, 66, 64, 67, 69, 66, 67	66·0±2·0
10 mM	50 µl 0·001 % brain homog. in 2 µM tannic acid	64, 67, 66, 70, 70, 72, 71, 72	69·0±3·0

enough to be efficacious. Also, the mechanism by which these particular molecules inhibit PrP-res formation in infected N2a cells is unclear and may not be replicated sufficiently *in vivo*. These results showed that inhibition in the infected N2a cells may not always correlate with anti-scrapie activity *in vivo*. Nonetheless, the scrapie-infected N2a assay remains a valuable initial screen for potential drugs because numerous compounds that have been identified as PrP-res inhibitors in scrapie-infected cells have proven to have at least some anti-scrapie activity *in vivo*.

Since many potent inhibitors of PrP-res formation *in vitro* are not efficacious against scrapie in animals, it is important to consider animal testing of inhibitors prior to clinical trials, especially in cases in which there might be negative side effects for the patient. Quinacrine, an anti-malarial drug used extensively in humans, was found to inhibit RML (Doh-ura *et al.*, 2000; Korth *et al.*, 2001) and later 22L (Kocisko *et al.*, 2003) mouse scrapie strains in cell culture. Based on its PrP-res inhibitory activity in cell culture, as well as its previous human use, testing of quinacrine in human TSE trials was strongly advocated, even in the absence of any supportive animal data (Korth *et al.*, 2001). However, in subsequent *in vivo* testing, quinacrine was ineffective in scrapie-infected mice (Collins *et al.*, 2002; Barret *et al.*, 2003) and, unfortunately, only transiently beneficial in some CJD patients, albeit with some liver toxicity (Kobayashi *et al.*, 2003; Nakajima *et al.*, 2004). Thus, although many inhibitors of PrP-res in cell culture are known, testing in TSE-infected rodents should help to select those with the most promise for human clinical trials.

When testing the efficacy of compounds against scrapie infection in an *in vivo* system there are many complex variables. Some of these include the dose, the vehicle, the dosing regimen, when to initiate and terminate treatment, the routes of compound administration and scrapie inoculation, the animal model and the TSE strain. Part of the difficulty in deciding on these variables is a lack of understanding of TSE pathogenesis. We have tried to select reasonable options from among these variables, but many others might be considered. Although none of the inhibitors tested herein was effective in our *in vivo* tests, we report the results of these expensive and time-consuming experiments in the hope that future work with potential anti-TSE therapeutics and prophylactics can advance beyond our particular approaches rather than repeat them.

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